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Interferons-α/β (IFN-α/β) and prolactin (PRL) are natural ligands relevant to breast cancer progression: IFN-α/β inhibits proliferation while PRL stimulates growth. The IFN-α/β antiproliferative action is mediated by STAT1 in a transcriptional holocomplex with STAT2 and ISGF3γ (~p48). #1 We investigated a negative regulator of ~p48 function, designated "TKO". Proteins in a cytosol fraction enriched for "TKO" were resolved by 2D PAGE. The ~p48-binding "TKO" (~22 kDa) was identified by depletion analyses. Microsequencing showed homology to alkylhydroperoxidase reductase C / thiol-specific antioxidants (AhpC / TSA). This superfamily includes human peroxiredoxins (Prx). Amino acid sequences in TKO peptide fragments most closely matched the Prx gene *PagA*. Primers were designed to amplify a unique portion of *PagA* for measurements of expression, and multiple breast cancer cell lines proved positive by RT-PCR. Polyclonal antibodies to synthetic oligopeptides also showed Prx expression. The full-coding sequence of *PagA* was tagged and cloned for transfer into insect cells with a baculovirus vector, and insect cell-expressed PagA was isolated for antibody production. #2 STAT1 serves as a transcription factor both for PRL and IFN-α/β. Tests for signal competition and potentially antagonistic "cross-talk" of PRL and IFN-α/β during STAT1 tyrosine phosphorylation nevertheless proved consistently negative in either well or poorly differentiated breast cancer cells. IFN-γ pretreatment augmented IFN-α/β antiproliferative activity, presumably due to an enhancement of ~p48 expression. Unexpectedly, it also enhanced STAT5 and STAT3 DNA-binding.

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INTRODUCTION

<u>Significance of interferons in breast cancer:</u> The functions of interferon (IFN) are clinically relevant to breast cancer growth and therapy. As endogenous cytokines, IFN-α/β and IFN-γ cooperate to regulate proliferation and differentiation of normal and malignant cells[1-3]. IFN-α/β can inhibit S-phase entry of cancer cells by a mechanism involving p21 at the START checkpoint in G_1 phase of the cell division cycle [4,5]. It is well documented that ionization radiotherapy or chemotherapy with conventional genotoxic agents increases the risk for development of secondary leukemia in women with breast cancer[6]. Treatment decisions for woman < age 50 years are especially complex.. Successful application of IFN-α/β in cancer treatment could avoid the side-effects and other non-specific damages to non-proliferating glandular tissues that result from genotoxic therapies. Despite promising results in pre-clinical models [7-9] , and evidence of positive IFN interactions with tamoxifen or retinoic acid [7,10,11], phase I/II clinical trials with IFN-α/β alone have fallen short of expectations[12]. A major aim of our investigations has been to elucidate a novel mechanism of cancer cell resistance which was detected at the level of IFN signal transduction and involves function of the key transcription factor Stat1.

<u>Prolactin (PRL) in breast cancer:</u> PRL is a principal mammotrophic growth factor. It can serve both as a mammary epithelial growth promoter, and as a lactogenic differentiation agent [13,14]. Up to 70% of human breast cancers are positive for PRL receptors[15,16]; and several established cell lines derived from breast cancers express abundant PRL receptors. Some of the latter can proliferate in response to PRL[16,17]. In rodents, excess PRL can initiate mammary gland nodular hyperplasia and neoplastic transformation[18]. In humans, association of prolactinoma with breast cancer has been noted. Although PRL classically originates from pituitary gland acidophil cells, endogenous production in human breast cancers has been reported[16,19].

<u>Stat1 is a key mediator of IFN biologic actions.</u> The expression of genes determining the principal biologic actions of IFN-α/β and of PRL can be enhanced or activated by the binding of Stat transcription factors to upstream promoter elements[2,20,21]. Stat proteins latent in the cytosol are activated by tyrosine phosphorylation[20-22]. Activated Stats translocate to the nucleus where they bind to DNA. Thus, Stats serve in a dual capacity as signal transducers and transcription factors[20-22]. For nuclear translocation, tyrosine phosphorylation and dimerization of the Stats is essential. For IFNs and PRL, the process of Stat phosphorylation is initiated by ligation of surface receptors. Type II cytokine receptors for IFNs become tyrosine phosphorylated by non-covalently associated and interdependent Janus tyrosine kinases (Jak1 or Tyk2 for IFN-α/β, Jak2 for IFN-γ)[21,22]. PRL signal transduction is initiated by ligation of type I receptors which typically are phosphorylated by Jak2[21-23]. As a consequence of association with the ligated and phosphorylated receptors, the Stats also become substrates for the Janus kinases (i.e. Jak1, Jak2, Tyk2) and thus undergo the critical tyrosine phosphorylation[20-22]. A total of seven Stat proteins have been identified and their biologic roles have been delineated by studies of knock-out mice[24]. Knock-out of Stat1 has shown that this transcription factor is essential to the antiviral and growth inhibitory actions of the IFNs[25,26].. Although PRL can activate Stat1, its major biologic actions are associated with activation of Stat5a and Stat5b[27,28]. Stat3 may play a complementary role in the physiologic functions of IFN or PRL[29], although the knock-out is lethal[24].

<u>TKO is a novel cytosolic inhibitor of IFN-α/β signals:</u> Activation of Stat1 through cell surface receptors for IFN-α/β (IFNAR) uniquely depends upon the co-activation of Stat2[30,31]. With IFNAR, Stat2 acts as an adaptor protein for Stat1 association and phosphorylation. Stat2 is not required for Stat1 activation induced

by receptors for IFN-γ, PRL or other growth factors[22]. In cells with IFNAR, activated Stat1 links with the activated Stat2 to form heterodimers (ISGF3α)[30,31.]. In turn, these associate with a potent DNA-binding protein ISGF3γ (~p48) to form a "holocomplex"[32]. The ~p48 dramatically increases the efficiency of gene transactivation[32]. Its turnover appears to be more rapid than for the Stat proteins, and it is upregulated by IFN-γ[32,33]. We have followed-up on a discovery that the signal transduction pathway for IFN-α/β is selectively interrupted in transformed cells by a negative regulator of ~p48[34]. This inhibitory factor was characterized as a ~p48-binding polypeptide and tentatively designated "transcriptional knock out factor" or "TKO". The negative effect on promoter element binding by the ISGF3 transcriptional holocomplex was related to blocking of potent DNA-binding by the ~p48 subunit[34]. Structurally, ~p48 is homologous to a family of IFN regulatory factors (IRF) which include a DNA-binding tumor suppressor IRF-1, an oncoprotein IRF-2, and a gene repressor ICSBP[32,35]. All are members of the *myb* family involved in cell proliferation and apoptosis[32].

Potential for cross-talk in the signal pathways of IFN and PRL: In addition to the IFNs, a spectrum of polypeptide hormones and growth factors, including multiple interleukins, platelet-derived growth factor, erythropoietin, growth hormone (GH), and PRL activate gene expression through the Janus kinase / Stat protein pathway[20-22,,36,37]. Studies with knock-out mice have shown that Stat1 is uniquely essential for the biologic action of IFN[25,26]; however PRL also is a potent inducer of Stat1 tyrosine phosphorylation[21]. Although not obviously essential to the known actions of PRL in knock-out mice[25,26], PRL Stat1 phosphorylation theoretically could modulate the biologic action of IFN-α. In breast cancer, such pathway such "cross-talk" might occur as a result of tumor-secreted PRL acting through an autocrine / paracrine feedback loop[19,38]. Stat5, which appears to be of minimal importance for IFN action[3], is an essential factor in the biologic action of PRL[13,21.]..

Purpose of project: This project was designed to investigate two independent sets of observations which appeared relevant to the antiproliferative function of interferon and role of Stat1 in breast cancer: (1) Original work from a collaborative study including the principle investigator had shown that a cytosolic protein from transformed cells ("TKO") negatively regulates the signal transduction pathway of IFN-α/β[34]. (2) Work reported by others had shown that human breast cancer cells autonomously secrete PRL[16, 19]. This raised the novel and significant possibility that PRL might modulate IFN-α/b actions due to a potent and common activation of Stat1.

Scope of research:

(1) Major efforts were directed toward characterization of the protein with TKO activity. Technical achievements included (a) purification of a candidate protein with TKO activity from cancer cell extracts; (b) amino acid (AA) sequencing of the candidate TKO protein and determination of its coding sequence; c) database analyses and matching of the TKO sequence to human anti-oxidant proteins in a highly conserved superfamily of alkylhydroperoxidase (AhpC) and thiol-specific antioxidant (TSA) enzymes; (d) selection of oligonucleotide sequences and design of primer pairs for RT-PCR to investigate expression of the TKO mRNA, based upon the sequence of homologous human genes known as peroxiredoxins (Prx); (e) testing of antibodies against synthesized oligopeptide sequences to investigate expression of Prx in breast cancer cells; (f) cloning of the coding region (g) N-terminal His-tagging, insertion of the full-length coding region into a baculovirus, and transfection of insect cells; (h) production of a specific His-tagged Prx and purification for antibody production.

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(2) Efforts to probe for evidence of cross-talk between the Jak / Stat signal pathways initiated by PRL and IFN-α/β. Technical achievements included (a) immunoprecipitations and Western blot analyses of Jak and Stat proteins extracted from lines of human breast cancer cells during respective cytokine and growth factor treatments; (b) electrophoretic mobility shift assays (EMSA) to compare Stat binding to selected oligonucleotide probes representing appropriate gene response elements for PRL (i.e. β-casein) or IFN-α/β (i.e. ISRE or IRF); © EMSA with anti-Stat antibodies to characterize changes in mobility of Stat-DNA complexes (supershift assays); and (d) proliferation assays to measure effect of the cytokine/growth factor co-treatments on cancer cell growth including cell counts, [³H]-dThd uptake, or MTT reduction.

BODY

Detection of a TKO activity in breast cancer cells: The Stat2-Stat1 heterodimer (ISGF3α) and the ~p48 subunit (ISGF3γ)comprising the DNA-binding ISGF3 holocomplex are separately capable of binding to DNA, but binding affinity of the Stat2-Stat1 complex is increased up to 25-fold when subunits are combined in the ISGF3 holocomplex[32]. The TKO activity was detected in cytosolic extracts of breast cells by means of EMSA. In the method employed, TKO inhibited the binding of Stat2-Stat1~p48 holocomplexes to oligonucleotide probes representing the ISRE sequence of an IFN- stimulated gene (ISG54K) or the "gamma-activated sequence" (GAS element) of the IRF-1 promoter[see 2,34,39,40]. Inhibition was detected by the absence of an EMSA "band shift ". The TKO activity proved particularly robust in cytosolic extracts from an established line of human breast cancer cells (Zr75-1)[41] (Fig. 2, 1995 Progress Report).

Enrichment of TKO activity in a cytosolic fraction. A cytosolic fraction enriched for TKO was prepared from extracts of breast cancer and cervical cancer cells. This involved a multi step process: dounce homogenization of cell pellets, sedimentation of nuclei and gradient separation of cell membranes, loading of the membrane-free supernatant over heparin-sepharose resin columns, collection and pooling of the resin flow-through, reloading onto a hydroxyl apatite column, and elution of a fraction with positive bioactivity in a sodium phosphate gradient. After processing, the specific activity for ~p48 binding, as determined by EMSA, was increased by ~10^4 fold Using this enriched fraction, proteins were resolved by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and a number of discrete spots in the 2D gel were detected by silver nitrate staining (Fig. 3, 1995 Progress Report). A candidate polypeptide in the expected molecular weight range of 20-30 kDa was identified in samples of two cell lines (ZR-75-1 and C33a). Its identity was confirmed by depletion analysis: the enriched cytosolic fraction was exposed to agarose beads coated with a synthetic N-terminal oligopeptide (125 AA) corresponding to the Stat-binding region of ~p48 ligand (the N-terminal oligopeptide was translated in vitro). This procedure succeeded in depleting the TKO activity and selectively eliminated the candidate polypeptide spot from 2D-PAGE preparations.

Bulk isolation of the candidate TKO protein for amino acid sequencing: For practical purposes, a line of cervical cancer cells (C33a) which showed evidence of potent TKO activity, comparable to that in the ZR75-1 breast cancer cells, could be grown in larger batches as suspensions. They were cultured to a density of ~1 x 10^6 cells/ml in volumes of 70 L; and a total of 8 batches were obtained for the pre-analytic cytosolic extraction and column separation procedures refined during this project (see 1996 Progress Report). Several single or combined samples representing ~300 ng of highly purified product with TKO activity that were thus obtained were subjected to peptide mapping. For each of these samples, the TKO "specific activity" was confirmed by EMSA.

Peptide mapping: Gels with samples of the resolved ~22 kDa candidate protein, were stained with amido black and transferred to nitrocellulose membranes. After washes with HPLC grade water, the membranes with transferred protein were wet frozen at -70°C and shipped in dry ice to the laboratory of Dr. Ruedi Aebersold in the Department of Microbiology at the University of Washington in Seattle. Tryptic digests for mass spectrometry were prepared by Dr. Steven Gygi. Preliminary database searches of tryptic digests by peptide mass matching were followed by mass spectrometric microsequencing of multiple peptide fragments with results: ighpapnfk; dislsdyk; qgglgpmniplvsdpk; tiaqdygvlk; adegisfr; glfiiddk; svdetlr; lvqafqftdk.

TKO sequence homology to genes coding an antioxidant superfamily: Public data base searches showed a close match of the sequenced TKO peptide fragments to aligned sequences which are characteristic of proteins in a superfamily of AhpC / TSA antioxidants. These enzymes have highly conserved in both prokaryotes and eukaryotes. Mammalian family members recently have been referred to as "peroxiredoxins" (Prx) [42-44]. Cys residues in positions 52 and 173 of the coding region are characteristic of AhpC / TSA (including Prx) and are related to the reductase function[45]. Open reading frames of genes in the AhpC / TSA superfamily typically code for proteins of ~ 200 AA[46-49]. This size was consistent with the estimated 22 kDa molecular weight of TKO; and a number of sequences found in our samples previously had been reported in Prx, including human NKEF, PagA, and rodent MSP23, OSF3 or MER5 [46-49]. Several TKO peptide sequences (apnfk, gglg, lvsd, glfiiddk, etlr, qafqf) were homologous to portions of AhpC / TSA from entamoeba or helicobacter [see 46]. The major and conserved function of the AhpC / TSA in prokaryotes and yeast is protection from free radical damages[see 50,51]. Mammalian Prx can be stress induced[52] and also appear to function in regulation of the tyrosine kinase c-abl[53] and the transcription factor NF-κB[43,54].

Best match to candidate TKO is PagA The Appendix Chart shows that sequences in the isolated protein with TKO activity were closest to those of a human Prx previously designated "proliferation-associated antigen" (PagA). The PagA was discovered in ras-transformed mammary cancer cells and by Prosperi et al.[46]. In comparison to other known human Prx, there was a significant difference of TKO from Prx1 (described as a "natural killer enhancing factor A" or "NKEFA")[48] in the region of AA 145-150 and there were multiple differences in TKO as compared to Prx2 ("natural killer enhancing factor B" or "NKEFB")[48].

RT-PCR amplification of mRNA from PagA or related genes: Based upon the published cDNA sequences for PagA and other human Prx[46-49], we prepared a cDNA representing a 195 base pair (bp) conserved region (bp 33 → 228) of the processed mRNA. Primers were: 5'-GGTGTCTGGTTAGTTTCTGC-3'(sense)and 5'-GACGGGATAGTGACTTTCGTTAC-3' (antisense). The amplified region encompassed codes for two of the oligopeptide fragments (ighpapnfk and dislsdyk) which were sequenced from the candidate TKO protein, and which are highly conserved throughout the AhpC / TSA superfamily. As one probe for product specificity, we synthesized a minus strand representing an intervening sequence characteristic of human Prx 5'-TGTTATGCCAGATGGTCAGT-3'(~vmpdgq).

Using RNA samples extracted from the ZR-75-1 breast cancer line previously shown to exhibit potent TKO function (Fig 2, 1995 Progress Report), RT-PCR amplified a specific cDNA product of anticipated size (Fig. 1, 1997 Progress Report). This product was generated by just 18 cycles of amplification and resolved sharply by DNA gel electrophoresis (ethidium bromide stain). For measurement, it was compared to equivalently amplified hypoxanthine phosphoribosyl transferase (HPRT) as a human "housekeeping" gene RNA control[55]. Using minus strand cDNA probe for quantization, expression of the TKO-related PagA gene was strong in four breast cancer cell lines: MCF-7, ZR-75-1, T-47D and Hs578T as well as in C33a cervical

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cancer cells and low passage foreskin fibroblasts. In accord with results of Prosperi et al.[46], expression appeared to be slightly greater in confluent cultures as compared to subconfluent cultures. Initial amplification, cloning and sequencing of multiple cDNA samples by the method of primer extension and fluorescent labeling showed no evidence for a consistent mutation of *PagA* in the human ZR-75-1 breast cancer cells or in a line of K562 leukemic stem cells which were a reported source of Prx II and the K562 cells were found by us to also express PagA.

Preliminary antibody detection of TKO-related protein expression in breast cancer cells: Two peptide sequences were selected for synthesis based upon the full cDNA sequence of presumptively related proteins in the human Prx family. These were used to elicit rabbit polyclonal antibodies. One of the synthetic sequences encompassed two adjacent peptide fragments found in the samples analyzed by Dr. Gyri: tiaqdygvlk and adegisfr (beginning at position 111 of the NKEF coding sequence). The other amino acid sequence selected was the C-terminal kpgsdtikpdvqkskeyfskqk which is well conserved in mammalian Prx genes[46]. Fig. 2 in the 1997 Progress Report showed results with whole cell SDS extracts resolved by SDS-PAGE (12%) and subjected to immunoblotting with polyclonal rabbit antibodies to the "111" peptide and "C-terminal" peptides. With two breast cancer cell lines (MCF-7 and ZR-75-1), and the cervical cancer C33a cell line, each antibody detected a ~22 kDa band consistent with the expected ~200 AA polypeptide (derived value from cDNA codons). The antibody to "111" peptide also localized a band at higher molecular weight position. The proteins were denatured in hot 1% SDS either with β-mercaptoethanol or dithiothreitol to exclude dimerization. The results thus may indicate the presence of a larger precursor protein.

Tests of anti-peptide antibodies on TKO-activity: The polyclonal antibodies to synthetic oligopeptide fragments described above were utilized in an effort to deplete TKO activity from cancer cell extracts prior to EMSA with ~p48. The EMSA employed a standard [^{32}P]-probe (Fcγ-GRR). No depletion of DNA-binding was noted. Since the rabbit antibody had been prepared against a linearized polypeptide rather than a native TKO-protein, such a result was thought to indicate that the tertiary protein configuration is critical to ligand binding. The next effort; therefore, was to express a complete human macromolecule of the Prx family using recombinant DNA technology. The ultimate objective was to stimulate antibodies to epitopes in native configuration. Presumably many of these epitopes would be shared with the TKO protein.

Expression of a recombinant TKO-related protein for antibody production: In order to expedite biosynthesis of an appropriate TKO immunogen, complementary cloning strategies were employed..

    (A) *GST-tagged protein:* Prior to complete matching of TKO with the coding sequence of *PagA*, project collaborators Dr. A. Larner and S. Vande Pol cloned the published coding sequence of NKEF which preliminary data suggested might be the candidate TKO. Primers were modified to include 5'-EcoR1 and XhoI-3' restriction enzyme cleavage sites. The full coding sequence was cloned into a pPCR-Script (Stratagene) vector, and amplified in E. Coli. The insert was excised and subcloned in the pGEX-4T-1 expression vector (Pharmacia) in frame for N-terminal expression with a glutathione S-transferase (GST) tag. The recombinant plasmid was used to transform of E. Coli DH 5α. After bacterial lysis, clarified supernatant was affinity purified with glutathione sepharose 4B and inoculated into rabbits. In preliminary tests, this antibody failed both for immunoprecipitations and Western blots of SDS-denatured proteins and for EMSA assays with native proteins in whole cell extracts. The presumptive problem was that GST is a 27 kDa protein which can modify antigenicity when ligated to a relatively small protein such as TKO. Although GST can in principle be cleaved free with a site-specific protease, this is a complex procedure of limited use. Therefore, production of a polyhistidine (His-tagged) candidate protein using a Baculovirus Expression

System (GIBCO BRL) was initiated. Since the His-tag is of relatively low molecular weight, the epitope was considered less likely to alter TKO immunogenicity.

(B) *His-tagged PagA:* Following the matching of "TKO" to *PagA,* primers for amplification of the full coding sequence were modified to include 5'-EcoRI and XhoI-3' restriction enzyme cleavage sites. Product obtained from ZR-75-1 cells was cloned into pPCR-Script and amplified in transformed *E.Coli* (DH 5α). Several colonies were streaked and screened with an appropriate [^{32}P]-cDNA internal probe to verify insertion of the *PagA* coding sequence. The insert was excised and subcloned into pFastBac HT donor plasmid (GIBCO method). DNA mini-preps and restriction endonuclease digestion verified correct insert orientation and the presence of H-tag in frame at the N-terminus. Commercial bacteria (*E.Coli*, Bac 10) previously transformed with baculovirus (Bacmid) and containing a disrupted *Lac7 gene* were transformed with the donor plasmid. A miniprep of high molecular DNA from this step was sequenced by PCR using fluorescent labeled dye terminators (Perkin Elmer Applied Biosystem 377 A) for confirmation. Lac7 disrupted (white) colonies (GIBCO protocol) containing the Bacmid with transposed DNA insert were identified by color and used for the final biologic amplification in SF9 insect cells. A mini-prep of high molecular DNA was used for transfection of SF9 cells with CellFectin reagent. Released baculovirus with the His-tagged DNA insert were used at low multiplicity of infection and plaque purified in SF9 cells. Expression of the tagged protein succeeded in transfected Hi5 insect cells as shown by separation of His-PagA from cytosolic extracts. The His-tagged protein was separated by binding to Ni-NTA agarose beads, column elution, eluate concentration, resolution of the proteins by SDS-PAGE, and detection of the His-PagA with an India HisProbe-HRP (Pierce) or by reaction with antibody to a synthetic oligopeptide representing the C-terminus of PagA. The same band was identified in preparative mini-gels stained with a methanol and acid-free Coomassie blue (Blue BANDit, Amresco, Solon, Ohio). The isolated band (Appendix Fig.1) was cut out and macerated for use in animal immunizations .

Lack of IFN-PRL signal cross-talk in human breast cancer cells: Three cytokine-responsive breast carcinoma cell lines of human origin (T47D, MCF-7 and BT-20) were tested for effects of IFN-α/β or PRL on Stat activation. Results indicated a cell-dependence of tyrosine phosphorylations with respect both to Janus kinase and Stat activations. IFN-α/β treatment produced an expected activation of the Janus kinases Jak1 and Tyk2 which are universally involved in Stat2-Stat1 tyrosine phosphorylations. Although Jak2 is the kinase primarily implicated in phosphorylation of Stat5 during PRL treatment of mammary epithelium, the experiments with breast cancer cells showed that PRL also activated Jak1 and Tyk2. Indeed, in highly PRL responsive T47D cells, the dose response and activation kinetics for Jak1, Jak2 and Tyk2 were comparable (see 1996 Progress Report).

Recent evidence points to Stat5 as an anti-apoptotic factor, as well as a differentiation factor during mammary development[47]. Although Stat3 and Stat5 can be activated by IFN-α/β in human lymphoma cells[3], they were activated only by PRL in the breast cancer cells, suggesting that the PRL- pathway was more efficient. Paradoxically, Stat3 has been implicated in hematopoietic cell growth inhibition[29], yet it was activated by growth promoting PRL rather than by the inhibitory IFN-α/β. This is consistent with cell-dependent differences in the roles of Stat proteins as reviewed elsewhere[20,21].

Downstream signal specificity of IFN and PRL: A significant upstream overlap in the activation of Stat proteins by type I IFNs and PRL, raised the possibility that PRL might interfere with IFN-induced Stat signals at the downstream level of DNA-binding or transcriptional activation. Formation of independent and qualitatively different Stat-DNA complexes was shown by electrophoretic mobility shift assays using probes representing relevant promoter elements in several of the well-established IFN-responsive genes[56] (see Fig.

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3, 1997 Progress Report). Since T47D cells express a relatively high number of PRL receptors per cell ($\sim$ 20×10^3), and exhibited the strongest responses to combined PRL and IFN-β, nuclear extracts from these cells were chosen to examine inducible Stat binding to [^{32}P]-oligonucleotide probes representing the ISRE of the ISG15 gene, the IRF1 GAS promoter element and the F$_c$$\gamma$R GRR element. EMSA disclosed significant differences in formation of the IFN-β and PRL-induced complexes, and showed that at the level of DNA-binding co-stimulation with PRL did not alter signals induced by IFN-β[56].

As anticipated, only IFN induced formation of a DNA-binding complex with the ISRE enhancer element (Fig. 3, 1997 Progress report) and this complex was completely supershifted or neutralized by antibody against either the Stat1 or Stat2 components of the ISGF3 holocomplex. Although IRF-1 is a tumor suppressor gene activated both by IFN-β and PRL, the IRF1 GAS complexes with Stats differed: PRL induced two separate complexes, a major slow migrating complex and a minor fast-migrating complex; whereas IFN-β induced a single and stronger fast-migrating complex[56]. Both fast-migrating complexes were clearly supershifted with anti-Stat1. The slow-migrating probably reflected a complex with Stat5b[38,56]. Results with the GRR probe generally proved similar, except that PRL induced only the slow-migrating complex which could not be supershifted with anti-Stat1. Additional evidence for independence of PRL and IFN signals was provided by differences in the DNA-binding complexes induced with the [^{32}P]-GAS sequence of the β-casein gene promoter. The β-casein gene is a known Stat5-regulated gene and the β-casein-derived GAS formed two distinct PRL-induced complexes which corresponded to two PRL-inducible and Stat5 positive complexes observed in rat Nb2 thymocytes[38,56]. These DNA complexes also were not supershifted by Stat5 antisera.

PRL fails to modulate the antiproliferative effect of IFN-β: Consistent with the downstream signal specificity, biologic autonomy of the IFN and PRL effects was maintained despite functional sharing of the critical Stat1 signal component. When T47D cells in mid-log growth were treated with IFN-β and/or PRL, a decrease in uptake of [^{3}H]-dThd caused by IFN-β was not diminished (Fig. 4, 1977 Progress Report). Similarly, no change was observed in a parallel set of experiments where the cells with pretreated with IFN-γ. These results were complemented by an MTT assay for metabolically viable cell mass. An important result of clinical interest was the finding that IFN-γ pretreatment consistently enhanced the action of IFN-α. This priming effect of IFN-γ also has been described in other experimental systems, but the clinical applicability has been overlooked in design of therapeutic protocols for breast cancer.

Independence of MAP kinase activation by PRL: The Erk MAP kinases have been implicated as critical regulators of Stat1 and Stat3 functional activation by serine phosphorylation[57]. Both PRL and type I IFNs can activate this pathway through _raf_. The possibility of crosstalk between PRL and IFN pathways at the MAP kinase level therefore was examined. Antibodies specific for the ser/thr-phosphorylated forms of Erk-1/2 (from Promega) were utilized for this analysis. Fig. 5 in the 1997 Progress report showed that despite marked Stat activation by IFN-α in the breast cancer system, there was no overt stimulation of Erk-1/2 phosphorylation in any of the cell lines tested. In contrast, PRL activated these MAP kinases to a variable degree both in MCF-7 and T47D cells. The absence of an effect in the line of BT-20 breast cancer cells may reflect high constitutive phosphorylation. In sum, there was no evidence of PRL and IFN cross-talk in the MAP kinase pathway and evidently no specific PRL modulation of Stat1 transactivation functions related to MAP kinase-dependent serine phosphorylations. IFN-γ also exerted no significant effect at this level.

CONCLUSIONS:

TKO is a Prx member of the AhpC / TSA superfamily: TKO, the cytosolic factor which negatively regulated transcriptional activation by the Stat2-Stat1 ~p48 holocomplex induced by IFN-α/β in breast cancer cells was identified as a protein in the AhpC / TSA superfamily. The *AhpC / TSA* genes are highly conserved in both prokaryotes and eukaryotes. The proteins are characterized by coding regions which express ~200 AA polypeptides. Cys residues at positions corresponding to 52 and 173 of linearized human homologues are characteristic and the sequence tfvcpte from human 49-55 has been conserved in helicobacter, yeast entamoeba, mouse, rat and human. Human and rodent genes in the AhpC superfamily have been designated "peroxiredoxins" (Prx)[42-45]. Comparing protein and derived nucleic acid sequences of TKO to some previously analyzed human genes *NKEFB (Prx II)*[48], *NKEFA (Prx I)*[48] and *PagA*[46], the closest match was *PagA*. Using primer pairs designed to amplify a Prx cDNA by RT-PCR, expression was detected in several breast cancer and other malignant cell lines. Multiple clones of cDNA, tested by the method of primer extension and fluorescent labeling indicated that the putative TKO sequence was consistent; however, the possibility of cancer specific mutations was not formally excluded.

Project delays in TKO purification, sequencing and analysis: Initial plans anticipated identification, cloning, expression and direct testing of a recombinant TKO protein using ~p48 binding in a functional EMSA. Testing of polyclonal or monoclonal TKO antibodies by abrogation of "TKO activity" also was projected. Pre-immune and immune rabbit sera were to be screened by EMSA either for functional activity in neutralizing or supershifting the complex of ~p48 with a [^{32}P]-GRR or [^{32}P]-ISRE probe. Any samples yielding a positive reaction were to be tested further by indirect or direct immunofluorescence with fresh or fixed TKO-positive breast cancer cells from the ZR75-1 line and C33a positive controls. These stages of the project could not be accomplished due to unexpected delays in purification and microsequencing of the candidate TKO protein. Recovery of pure TKO in nanogram quantities required mass culture techniques and more than one year of material collection. With the small quantities finally obtained, several laboratories were unable to provide useful data (see Progress Reports for 1996, 1997). Although the final identification as a human Prx was very important and potentially significant to our understanding of IFN-resistance in breast cancers, there were a number of further problems requiring experimental work. A close identity of AA sequences in the coding region made separation of TKO from Prx I and Prx II difficult to prove. The close match to PagA was based upon analysis of the AA sequence from 140-150 and the N-terminal zone. Primers could not be found to separate *Prx I* and *PagA*, but multiple cloned samples sequenced by PCR using fluorescent labeled dye terminators consistently showed recovery of the *PagA* sequence in ZR-75-1 breast cancer and K562 leukemia cell lines. Further work nevertheless is needed to assess whether the presumptive sequence of the TKO-related PagA gene in cancer cells is always identical to the sequence in non-neoplastic cells (see below).

Preparation of purified recombinant PagA: The failure of a GST-tagged human Prx protein to generate antibodies that functioned in EMSA, immunoprecipitations or Western blot was the impetus to production of a His-tagged PagA in insect cells. This was a far more complex process than preparation of a GST-product and purification of the His-PagA from insect cell cultures required almost one year of trial and errors with various buffers fro extraction and use of bead separations by molecular size or affinities. As a result, native protein is only now for antibody production.

Rabbit antibodies to synthetic peptide fragments characteristic of human Prx identified a relevant antigen of 22 kDa in breast cancer cell lines; however, these antibodies were not capable of

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immunoprecipitations or useful for Western blots. They also failed to block TKO function binding to ~p48 as determined by EMSA.

In many experiments with the rabbit antibodies, a dimer of 44 kDa was identified. Based upon recent crystallographic work with a rat Prx related to PagA[45] , it is likely that this represents the oxidized form of PagA during involvement in peroxidation catalysis. Experiments with a peptide competitor or with affinity purified antibodies to the entire PagA or large subunits of PagA should resolve these questions.

Potential significance of TKO / Prx identification in human breast cancer: The finding of TKO activity   in breast cancer cells is potentially highly significant. Not only is TKO a factor implicated in the regulation of IFN-α/β antiproliferative function, but related Prx proteins have been shown to bind NF-κB or c-abl in other experimental models. Thus, Prx/TKO may have important roles in cancer cell proliferation, differentiation or survival under stress[52,58,59]. The functional relationship of TKO activity to the levels of *PagA* or related gene expression needs to be assessed, and availability of an antibody useful for immunoblotting would be valuable.

Non-competitive roles of PRL and IFN in Jak-Stat signal transduction and growth inhibition: Type I IFNs inhibit the growth of mammary epithelial cells and serve as adjuvant agents in breast cancer therapy. Conversely PRL is one of the principal mammotrophic factors and a tumor promoter in rodent mammary gland. Despite these contrasts in biologic impact, type I IFNs and PRL signals both can induce tyrosine phosphorylation of Stat1, a transcription factor that is critical to the function of IFN. In addition, serine phosphorylations been implicated in Stat1 signals. Combined effects of IFN-α/β and PRL were consistently additive and revealed no negative cross-talk due to competition for Stat1. Results were consistent with a role of Stat2 as a docking factor for Stat1 in relation to the IFNAR[30], but not necessary for interaction of Stat1 with the PRL receptor.

Despite the overt disparities in regulatory actions of IFN-α/β (growth inhibition) and PRL (growth stimulation and differentiation) , no evidence of a competition between these signal pathways emerged at the level of Stat1 activation (the crucial factor in IFN-α/β biological action). In fact, the 1996 Progress Report (Fig. 3) showed an additive effect of PRL on IFN-α-induced Stat2-Stat1 tyrosine phosphorylation in T47D cells or IFN-β induced Stat2-Stat1 tyrosine phosphorylation in MCF-7 cells. Present work indicates that endogenous local or systemic production of PRL in patients with breast cancers would not be a significant factor in modulating the therapeutic actions of exogenously administered type I IFNs.   While the MAP kinases have an established role in promoting cell growth and oncogenesis, induction of Erk-1/2 by PRL failed to counteract the growth-inhibitory effect of IFNs when tested in T47D and MCF-7 cells.

Potential utility of IFN-γ in breast cancer therapy: An important finding was the observation of increased IFN-α signal strength and anti-proliferative activity in the presence of IFN-γ[56]

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Figure 1 - Resolution of His-PagA on preparative 12% acrylamide mini-gel.
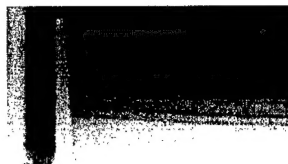
Left lane molecular weight markers counting from the bottom represent 13, 20 and 26 kDa respectively. The His-PagA appears as the separate dense band between 20 and 26 kDa.

Chart

The published amino acid sequences of PagA, NKEFA (Prx I) and NKEFB (Prx II) are compared. The residues coded pink were directly sequenced in samples of purified TKO protein and proved identical to comparable regions of PagA. A significant difference from NKEFA was noted at positions 145-150 where NKEFA exhibits an unusual double cystein. Significant differences from NKEFB were found in multple sites from positions 60-95 (not shown) and at positions 20-30 (note A and EVK).
Multiple other differences were noted in the regions of 105-130. The portions of TKO which were directly sequenced included several major conserved regions of Prx underlined in red. These include papnfk, dislsdy, glfiiddk, vndlpvg and rsdvdetlr.

"NYEFA"

PAGA


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  1 MSSGNAKIGHPAPNFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTF 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 MSSGNAKIGHPAPNFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTF 50

 51 VCPTEIIAFSDRAEEFKKLNCQVIGASVDSHFCHLAWVNTPKKQGGLGPM 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 VCPTEIIAFSDRAEEFKKLNCQVIGASVDSHFCHLAWVNTPKKQGGLGPM 100

101 NIPLVSDPKRTIAQDYGVLKADEGISFRGLFIIDDKGILRQITVNDPPCC 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 NIPLVSDPKRTIAQDYGVLKADEGISFRGLFIIDDKGILRQITVNDPPVG 150

151 RSVDETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVPKTKEYFSKQK* 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 RSVDETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK* 200
```

NYEF B

NYEF A

- PAGA

(C Term)

P = proline
Q = glutamine
T = threonine
S = serine

pink = sequenced by Diego
yellow = for peptide M
green = probe region
red = major conserved region

PUBLICATION / ABSTRACTS

Rui, H., Erwin, A., Farrar, W.L., Larner, A.C., Grimley, P.M. Interaction of prolactin and interferons α/β on JAK/STAT signaling in human breast cancer lines. J. IFN Cytokine Res. 15:S167, 1995

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